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Contents

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RAPID METHOD FOR ESTIMATION OF GLYCEROL IN FERMENTATION SOLUTIONS[1]

BY MARGUERITE LAMBERT AND A. C. NEISH

Abstract

The glycerol is quantitatively oxidized to formaldehyde by periodic acid, using a five minute oxidation period to minimize oxidation of glucose. The iodate and periodate are reduced to iodide by a large excess of sodium arsenite and the formaldehyde then determined directly in the oxidation mixture, by the color reaction with chromotropic acid. The precision of the method is about $\pm$ 2% in the range 2 to 8 p.p.m. of glycerol. The error due to glucose, in quantity equal to the glycerol, is only 2.5 to 5.0% and can be easily corrected for when the glucose content of the solution is known. One worker can analyze 40 to 60 samples per day.

Introduction

The production of glycerol from glucose in fairly good yields by organisms such as the yeasts and some bacteria suggests that even better organisms might be found if hundreds of strains were tested. One drawback to attempting such a survey has been the lack of a rapid analytical method for determining glycerol in the presence of residual sugars and 2, 3-butanediol. A method has been developed for this purpose and is described in this paper.

In selecting a method it was decided that a procedure based on measurement of the formaldehyde formed by periodate oxidation would probably be the best since glycerol gives a higher yield of formaldehyde than any compound, except ethanediol which is not likely to be found in fermentation solutions. A colorimetric method for determination of the formaldehyde should be more suitable for running large numbers of determinations than a gravimetric or volumetric procedure. Desnuelle and Naudet (3) have adapted Schryver's test (9) to the colorimetric determination of formaldehyde in periodate oxidation mixtures. This is based on measurement of the red color obtained when a formaldehyde solution is mixed with freshly prepared solutions of phenylhydrazine, potassium ferricyanide, and concentrated hydrochloric acid at 0°C. The present method is based on the use of chromotropic acid (1, 8-dihydroxynapthalene-3, 6-disulphonic acid) which has been shown by Eegriwe (4) to be a specific reagent for formaldehyde in the presence of other aldehydes. It has been used by Boyd and Logan (1) for the quantitative determination of

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formaldehyde formed on periodate oxidation after separation of the formaldehyde from the excess periodate and iodate by distillation. In the present work this distillation step has been eliminated by reduction of the periodate and iodate, to iodide by an excess of sodium arsenite. This is necessary since both iodate and periodate interfere, causing a deep color in the blank. Corcoran and Page (2) have recently described a very similar method which was used for the determination of mannitol in plasma and urine. They used stannous chloride in place of arsenite. Arsenite solutions have the advantage of being stable while the stannous chloride solution must be freshly prepared and standardized each day. 2,3-Butanediol does not interfere since it gives no formaldehyde while the interference due to glucose is minimized by using a short oxidation time (2). The method for developing the color is the same as that used by MacFadyen (6) for determination of formaldehyde in biological mixtures.

Method

Reagents

(1) Sulphuric acid (10 *N*)—280 ml. of concentrated sulphuric acid added cautiously to 600 ml. of water and diluted to one liter when cool.

(2) Sodium periodate (0.1 *M*)—11.5 gm. of periodic acid dissolved in 450 ml. of water and neutralized with 1 *N* sodium hydroxide until faintly acid to methyl red.

(3) Sodium arsenite (1 *M*)—22.5 gm. of sodium hydroxide and 50 gm. of arsenious acid (As_2O_3) dissolved in 500 ml. of water.

(4) Chromotropic acid reagent (after MacFadyen (6))—Dissolve 1 gm. Eastman's 1, 8-dihydroxynaphthalene-3, 6-disulphonic acid (chromotropic acid) in 100 ml. of distilled water and filter. Add 300 ml. of concentrated sulphuric acid to 150 ml. of water, cool, and add to sulphonic acid solution to make 500 ml. Store in brown glass stoppered bottle and prepare fresh every two to three weeks.

Procedure

A sample of the fermentation solution containing 0.2 to 0.8 mgm. of glycerol and not exceeding 20 ml. in volume is pipetted into a 100 ml. volumetric flask provided with a glass stopper. Sufficient distilled water is added, if necessary, to make the volume 20 ml. and then 1 ml. of 10 *N* sulphuric acid. Five milliliters of the sodium periodate is added, with mixing, to each flask of the series and exactly five minutes after addition of the periodate to the first flask, 5 ml. of the arsenite solution is added in the same way so that five minutes will elapse between the addition of periodate and arsenite to each flask of the series. A 5 ml. hypodermic syringe, used without a needle, is very useful for adding these reagents. Twenty flasks, including a blank, may be run in each series. Ten or twenty seconds after addition of the arsenite, iodine appears in the solution and then fades. After 5 to 10 min. the contents of the flask is made to the mark, mixed, and 1 ml. pipetted into a 25 by 200 mm. Pyrex test tube.

Ten milliliters of the chromotropic acid reagent is added with mixing and the tubes heated for 30 min. in a boiling water bath in a rack with sheet metal walls designed to prevent direct light from falling in the tubes while they are being heated. The tubes are then cooled in water and the per cent transmittance determined at λ570 in a Coleman Model 6 spectrophotometer using 25 mm. round cuvettes. Alternatively the color density may be read using an Evelyn colorimeter with a 540 M filter. A blank must be run with each set in order to obtain the correct 100% transmission setting; it should represent the same amount of periodate and arsenite as was added to the other solutions. A calibration curve is made from pure glycerol solutions. An 0.5% aqueous solution of glycerol is prepared from C.P. glycerol and standardized by measuring its periodate reducing value (5, 8). This is kept in a refrigerator and portions of it suitably diluted as needed for calibration of the method.

Results and Discussion

When the method is applied to pure solutions of glycerol the Beer–Lambert law is obeyed as shown in Table I. These data give a straight line when log % transmittance is plotted against the concentration of glycerol. Calibration curves run at different times using different batches of reagent agree closely. However, it is recommended that the curve be checked from time to time.

TABLE I

CALIBRATION CURVES FOR GLYCEROL DETERMINATION

Evelyn colorimeter Filter 540 M, 19 mm. cuvette		Coleman Model 6 Spectrophotometer λ570, 25 mm. cuvette	
γ glycerol per tube	% transmittance	γ glycerol per tube	% transmittance
3.70	63 1/2	1.00	85
4.00	60 3/4	1.85	74 1/2
7.40	40	2.50	67
8.00	37 1/4	3.70	55
11.10	25 1/2	5.55	41
12.00	22 3/4	7.40	31
14.80	15 3/4	9.25	23
16.00	14 1/4	10.04	20 1/4

Since fermentation solutions nearly always contain some residual carbohydrate it is necessary to correct for any possible errors from this source. The carbohydrate content of the fermentation solution is always measured first, usually by the color given with anthrone (7) in order to see if the organisms are capable of fermenting glucose. Consequently a correction can be applied for the formaldehyde given by the residual glucose. If the oxidation conditions are chosen so that the glucose is oxidized completely to formic acid and formaldehyde it will give 0.256 as much formaldehyde as an equal weight of glycerol.

However, this correction can be reduced to about one-tenth of this value by taking advantage of the fact that glycerol oxidizes much more rapidly than glucose. Table II shows that the fraction of glucose oxidized is independent of its concentration, under the conditions employed, and that almost four

TABLE II

RATE OF OXIDATION OF GLUCOSE TO FORMALDEHYDE

Oxidations run in parallel at room temperature (24° to 26°C.) using the same conditions employed in analysis of solution but varying the amount of glucose per flask and the time of oxidation.

Time, min.	Yield of formaldehyde as % of theoretical		
	2.50 mgm. of glucose	1.00 mgm. of glucose	0.50 mgm. of glucose
5	12.6	14.1	12.0
15	24.0	23.8	19.2
60	60.8	65.2	59.1
124	89.6	93.0	91.2
182	96.6	99.5	99.3
241	99.9	99.5	99.3

hours is required for the reaction to go to completion as measured by formaldehyde formed. On the other hand glycerol is completely oxidized in five minutes under the same conditions. Consequently if the reaction is stopped after five minutes, by the addition of arsenite, the error due to glucose is quite small, being in the range of 2.5 to 5% depending on the temperature (see Table III). Hence, it is hardly necessary to make a correction unless the concentration of glucose is more than one-half that of the glycerol and even if there is five times as much glucose as glycerol the correction is small enough so the glycerol can be determined with reasonable accuracy if the proper correc-

TABLE III

EFFECT OF TEMPERATURE ON RATE OF OXIDATION OF GLUCOSE TO FORMALDEHYDE

Oxidations run under same conditions as used in analysis (five minute oxidation) but with temperature controlled within ± 0.25°C.; 2.50 mgm. of glucose per flask.

Temperature, °C.	Yield of formaldehyde, % of theory	% correction for glucose* in glycerol determination
19.5	9.4, 9.8	0.0246
23.5	12.0, 12.8	0.0317
24.5	13.9, 13.9	0.0356
26.5	16.0, 16.6	0.0416
30.5	19.8, 20.1	0.0510

* *Multiply the per cent glucose in the solution by factor in this column and subtract the result from the per cent of glycerol found to obtain the true glycerol content.*

tion is applied. This is demonstrated by the results in Table IV, which show that the error from this source is not likely to exceed 5% unless the concentration of glucose is more than eight times that of the glycerol. The most

TABLE IV

ANALYSIS OF SYNTHETIC MIXTURES OF GLUCOSE AND GLYCEROL

A five minute oxidation period was used, as described in the method.

No.	Added (γ per ml.)		Glycerol found (γ per ml.)		% recovery of glycerol
	Glucose	Glycerol	Apparent	Corrected for glucose	
1	25.0	2.24	3.00	2.13	95.1
2	25.0	2.24	2.96	2.09	93.5
3	25.0	3.36	4.34	3.47	103.2
4	25.0	3.36	4.22	3.35	99.8
5	25.0	3.36	4.46	3.49	103.8
6	25.0	3.36	4.46	3.49	103.8
7	25.0	4.48	5.29	4.42	98.9
8	25.0	4.48	5.29	4.42	98.9
9	25.0	5.61	6.58	5.61	100.0
10	25.0	5.61	6.61	5.64	100.5
11	10.0	3.36	3.80	3.41	101.7
12	10.0	3.36	3.83	3.44	102.3
13	10.0	5.61	5.99	5.60	99.8
14	10.0	5.61	6.05	5.66	101.0
15	5.0	3.36	3.58	3.39	100.9
16	5.0	3.36	3.58	3.39	100.9
17	5.0	5.61	5.80	5.61	100.0
18	5.0	5.61	5.80	5.61	100.0

accurate method of obtaining the right correction is to oxidize a glucose solution of known concentration (2.5 mgm. per flask) in series with the solutions being analyzed. Some experiments were tried using copper–lime treatment to remove glucose but this gave variable results with about 10% apparent loss of the glycerol.

There do not seem to be any substances in yeast fermentation solutions which markedly interfere in this method since added glycerol can be determined with less than 4% error (Table V).

TABLE V

RECOVERY OF GLYCEROL ADDED TO SOLUTIONS FERMENTED BY YEAST

Yeast solution No.	γ glycerol per ml. originally	γ glycerol per ml. added	γ glycerol found	Added glycerol recovered	% recovered
1	84	251	335	251	100.0
	84	502	578	494	98.4
2	261	251	504	243	96.9
	261	502	745	484	96.5
3	300	251	555	255	101.8
	300	502	795	495	98.8

The reproducibility of the results obtained by this method agree favorably with those usually obtained by colorimetric methods. Analysis of a number of solutions fermented by *B. subtilis* in triplicate (Table VI) shows that the errors caused by the various manipulations are not likely to exceed 2.5%.

TABLE VI

REPRODUCIBILITY OF METHOD IN ANALYZING *B. subtilis* FERMENTATION SOLUTIONS

Five solutions containing 5% glucose and 0.5% yeast extract were fermented by *B. subtilis* (Ford's type) and each one analyzed three times for glycerol on three successive days by the method described in this paper. Solution stored at 2°C. between analyses.

Fermentation solution No.	% glycerol found			Average % glycerol
	1st day	2nd day	3rd day	
1	0.772	0.783	0.795	0.783
2	0.783	0.818	0.810	0.804
3	0.805	0.828	0.810	0.814
4	0.845	0.858	0.845	0.849
5	0.718	0.738	0.738	0.731

Maximum deviation from average ± 0.019 or 2.4%
Mean deviation from average ± 0.009 or 1.2%

It is difficult to be sure exactly what the glycerol content of a fermentation solution is since none of the methods for determining it are absolutely specific. The colorimetric method described in this paper was compared with another method for determining glycerol which is based on measurement of the periodate reducing value of the solution after extraction of 2, 3-butanediol and some other interfering substances by ether (8). The results obtained (Table VII)

TABLE VII

COMPARISON OF METHODS FOR DETERMINATION OF GLYCEROL IN FERMENTATION SOLUTIONS

Solutions containing 5% glucose and 0.5% yeast extract were fermented to completion. The amount of glycerol produced by yeast was varied by controlling the pH at different values. Glycerol was determined directly in the fermentation solutions by the method described in the paper, and also by the method used previously for analyzing *B. subtilis* fermentations which is based on the determination of the periodate consumed after removing 2, 3-butanediol by ether extraction (8). A correction was applied for the small amount of residual glucose, or in the use of the volumetric method, it was removed by copper-lime treatment.

Solution fermented by	% residual carbohydrate (as glucose)	% glycerol found by	
		Colorimetric determination of formaldehyde	Iodometric titration of excess periodate
Yeast	0.160	0.166	0.169
Yeast	0.184	0.196	0.185
Yeast	0.250	0.399	0.372
Yeast	0.078	0.402	0.379
B. subtilis (Ford's type)	0.056	0.872	0.880

show reasonably good agreement between the two methods, considering that they are based on measurement of different things. In the first case the formaldehyde formed by periodate oxidation is measured and in the second case the periodate reduced is determined. The first method is the most specific but certain substances, such as serine or mannitol (2) (or other sugar alcohols) will also be measured by it. However, serine is not likely to be present in high enough concentration compared to the glycerol to be much of a source of error. On the other hand, the sugar alcohols may be present in high concentration if they are formed by reduction of the glucose, a reaction which is known to occur with some organisms. Hence it is not certain whether glycerol or some other compound in the same homologous series is being measured unless it is identified by some other means. Since the ratio of moles of periodate reduced to moles of formaldehyde formed is 1.0 for glycerol and 2.5 for an acyclic hexitol it is possible to be more certain by measuring both these quantities when an organism giving substantial amounts of 'apparent glycerol' is found. However, the only way to be positive that an organism is producing glycerol is to isolate it in a pure form and prepare crystalline derivatives. The analytical method described in this paper is intended to enable the selection of cultures which appear worth investigating further. Some recent experiments by one of us (A.C.N.), which will form the subject of a future communication have shown that glycerol may be separated from interfering substances by partition chromatography on celite–water columns.

Milder conditions might have been chosen for the oxidation procedure used; for example, the sulphuric acid might be omitted. However, acid conditions are needed for the reduction of the iodate by arsenite. Possibly this reaction is catalyzed by iodide although none is added other than that present as an impurity in the reagents. A large excess of the arsenite solution is used because of the reversibility of the reaction between arsenite and iodine. The blank has some color which can be reduced by using less periodate. This is possible in experiments on pure substances but it was felt advisable to use a large excess on fermentation solutions.

The method is sufficiently rapid to be used in screening large numbers of micro-organisms. One worker can analyze 40 to 60 solutions in a day.

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MERCURY 1850 Å AND XENON 1470 Å RESONANCE LAMPS AS ULTRAVIOLET SOURCES FOR PHOTOCHEMICAL STUDIES[1]

By J. R. Dacey[2] and J. W. Hodgins[3]

Abstract

A mercury resonance lamp is described whose output is 6×10^{15} quanta per second of radiation of wave length 1850 Å. The radiation is emitted through a window of synthetic sapphire. The lamp is of simple construction and gives consistent and trouble free operation. A xenon lamp is also described which emits 1×10^{16} quanta per second of radiation below 1470 Å. The construction, method of calibration, and some of the operating characteristics of the lamp are given.

Introduction

During an investigation of the photosensitized reaction of some fluorocarbons, high energy light sources were required because of the strength of the carbon–fluorine bond. When the common mercury 2537 Å resonance lamp failed to produce any reaction, a higher energy source was constructed which gave the 1849.6 Å resonance line of mercury corresponding to 154.5 kcal. per einstein. Although lamps giving this radiation have been used before (8, p. 39), no detailed description of their construction has been found. Workers using this wave length have made use of thin blown quartz as material for windows for their lamps and reaction cells. Fluorite and lithium fluoride have also been used for windows for transmission of short ultraviolet. Freed, McMurray, and Rosenbaum reported (4) that plates of artificial sapphire (white sapphire or corundum, Al_2O_3) 1/16 in. thick were transparent to at least 1435 Å. It was thus decided to construct a lamp such that the reaction cell and the lamp had a common window of artificial sapphire. The corundum window had the advantage of being mechanically strong, resistant to thermal shock, chemically inert, and almost impossible to scratch. Thus a lamp with such a window was much more robust than one using windows of thin quartz or plates of fluorite or lithium fluoride.

For radiation of still higher energy, it was decided to construct a light source emitting the 1470 Å resonance line of xenon. The first xenon lamp was described by Harteck and Oppenheimer (6); it was filled to a pressure of 20 mm. of neon and 100 μ of xenon, and operated at about 90 v. and 50 amp. Groth (5) achieved a tenfold increase in intensity by applying a strong magnetic field (4000 to 5000 gauss) near the window, which increased the potential drop in that area, and, by means of the Zeeman effect, reduced the amount of reabsorption. A quartz body was used for this lamp, owing to the large quantity of heat generated, and the light was emitted through a fluorite window. The intensity was very high (2×10^{16} quanta per second),

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Contribution from the Defence Research Chemical Laboratories, Ottawa, and the Royal Military College of Canada, Kingston, Ont. The laboratory work was carried out at the Defence Research Chemical Laboratories, and is reported as D.R.C.L. No. 36.
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and its life was about 100 hr. This lamp, while giving intense emission, had the disadvantage of running very hot, making it difficult to assign a value to the reaction temperature. When studying the xenon photosensitized decomposition of hydrogen, Calvert (2) used a high voltage xenon discharge lamp operating at about 1600 v. and 70 to 100 ma. A fluorite window was also used in this lamp, which had a low output of about 2×10^{13} quanta per second.

The basis of choice of the xenon discharge lamp for the study of the photochemical reactions of carbon tetrafluoride was twofold. First, short ultraviolet was necessary, since the strength of the carbon–fluorine bond was known to be high, and the 1470 Å line of xenon has an energy of 194.4 kcal. per einstein. Second, since xenon is so inert chemically, there is little likelihood of the formation of xenon fluoride when studying the xenon photosensitized reactions. Evaluation of the quantum output of such a lamp was not difficult, since Groth (5) had carefully determined the quantum efficiency of the photodecomposition of carbon dioxide by xenon resonance radiation. Taking into account the recombination of oxygen with carbon monoxide, he found the quantum efficiency of the process to be 0.98.

The first xenon lamp constructed in the present investigation had a lithium fluoride window, and ordinary neon sign electrodes about 10 in. apart. Because of the geometry, operation at a low voltage and a high current was impossible. When the lamp was operated at high voltage on a neon sign transformer, the output proved inadequate, owing probably to reabsorption of radiation by unexcited xenon atoms in the space between the discharge and the window. The second lamp, described in a following section, made use of a very thin quartz window, and gave a satisfactory output of short wave ultraviolet. The two principle resonance lines of xenon are at 1470 Å and 1295 Å. Since a vacuum fluorite spectograph was not available for photographing the discharge, the assumption has had to be made that the output of this lamp is preponderantly 1470 Å, since it is thought unlikely that the 1295 Å line would be transmitted through the thin quartz.

The Mercury Resonance Lamp

The 1850 Å lamp is illustrated in Fig. 1. The body consisted of two coaxial Pyrex tubes, T_1 and T_2 fitted with standard iron neon sign electrodes. The upper end of the outer tube was a 29/42 standard taper male joint, which was shortened so that the diameter at the end was 26 mm. The end of the lamp was closed by a window of synthetic sapphire* (diameter = 25mm., thickness = 1mm.) cemented to the end of the tapered joint with Benolite, a thermosetting plastic cement. The end of the inner tube was only ½ in. from the window, in order to minimize absorption of radiation by unexcited mercury atoms. The reaction space was formed by tube T_3, the female portion of the tapered joint, so that the lamp and the reaction cell have one

**Fabricated by Sapphire Products Division of the Elgin Watch Company, Aurora, Illinois.*

common window. Grease is undesirable in the reaction space, so, in use, a seal was effected by filling cup *C* with mercury. The mercury cup was cooled by water coil *D*.

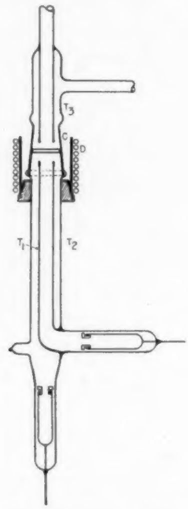

FIG. 1. *Mercury 1850 Å resonance lamp.*

The filling of the lamp, after outgassing and "burning in" of the electrodes, consisted of a drop of mercury, and neon at 7mm. pressure. It was operated on alternating current at 100 ma. from a 6000 v. current limiting transformer. The neon had its usual function of carrying the current until the pressure of mercury vapor was sufficient for the mercury discharge to occur.

A Moll large surface thermopile was used to measure the energy output from the lamp. During calibration, a fiber tube was used to connect the lamp to the entrance of the thermopile, so that a small flow of any gas could be circulated between the lamp window and the surface of the thermopile. The thermopile was carefully insulated, and the assembly set up in a darkroom, with the thermopile leads connected to a potentiometer outside the darkroom, at a distance of about 10 ft. The calibration of the thermopile had been checked previously by comparing its values with those obtained by uranyl oxalate actinometry on the radiation from a mercury 2537 Å lamp.

The total radiation from the lamp was first measured with purified nitrogen circulating in the fiber tube. After the lamp had been on for sufficient time to become stable, a measurement was made of the thermopile e.m.f. The lamp was turned off, and a second reading taken immediately. The difference in these readings was taken to be the total output of the lamp, both visible and ultraviolet. An average of a series of such readings was taken. Then a series of readings was taken of the energy output, with dry ammonia circu-

lating between the lamp and the thermopile. Since ammonia strongly absorbs 1850 Å (7) but not 2537 Å or visible light, the difference between this series and the previous one gives the energy of the 1850 Å radiation. The intensity of 1850 Å radiation was obtained at three distances from the lamp, and, from these data, the intensity close to the window was calculated. The output of the lamp was thus found to be 6×10^{15} quanta per second.

The Xenon Resonance Lamp

The xenon resonance lamp and reaction cell are illustrated in Fig. 2. The central tube through which the radiation was emitted to the gas sample was made by drawing out quartz tubing until its wall had an average thickness

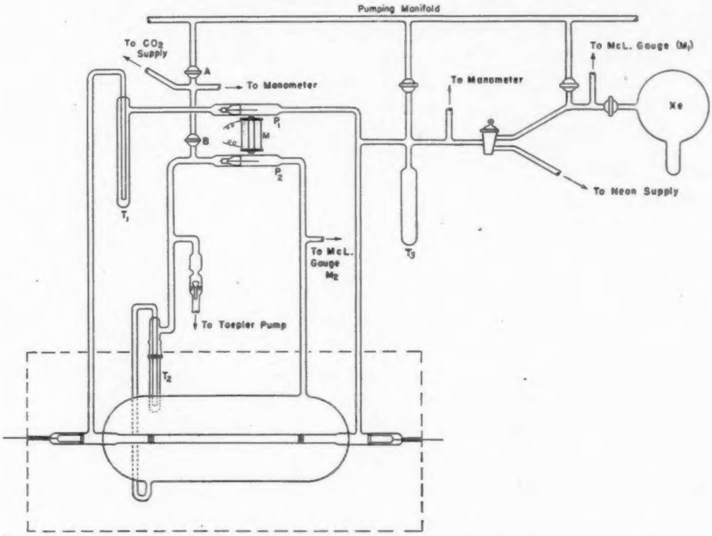

FIG. 2. *Xenon 1470 Å resonance lamp and reaction system.*

of about 0.05 mm. This "window" was 20 cm. long and 1 cm. in diameter and was joined to the electrode chambers through quartz-to-Pyrex graded seals. The reaction space surrounded this central discharge tube and had a diameter of 10 cm. The lamp was operated on 60 cycle current from a 5000 v. transformer whose primary windings were connected through a 16 ohm rheostat to a transformer whose voltage was continuously variable from 0 to 120 v.

The method of filling the lamp is apparent from Fig. 2. The diagram shows a circulation system for the lamp filling. In early experiments, quite low xenon pressures were used (about 50μ) and, after about an hour's operation, the discharge would cease. It was found that continuous operation could

be achieved by condensing an excess of xenon in trap T_1, and maintaining it at a temperature corresponding to a vapor pressure of 50μ ($-$ 190 °C.). Subsequent experiments showed that the electrodes were adsorbing a considerable quantity of xenon, and that satisfactory operation could be achieved by using a higher xenon pressure, after exhaustive outgassing of the electrodes and "burning in" of the lamp. Thus best operation of the lamp was obtained when it was filled with xenon at 0.1 to 0.5 mm. pressure and neon at about 5 mm. pressure. The neon served to conduct heat from the electrodes. An operating current of 120 ma. was commonly used. The lamp was kept immersed in a water tank (shown in broken lines in Fig. 2.)

The circulating pumps (P_1, P_2, in Fig. 2) used in this apparatus are of interest. Each consisted of a brass boss gripping a spring steel reed of such length that, when plucked, its frequency was a harmonic of 60 c.p.s. The brass frame was located in a 2 cm. glass tube and held in place by "dimpling" the glass into an indentation in the frame. The reeds were vibrated by means of a magnet (*M*, Fig. 2) operating on 60 cycle current. Although the rate of circulation at low pressures is not known, at atmospheric pressure the rate was about 3 liters per minute.

The xenon lamp was calibrated by measuring the rate of decomposition of carbon dioxide by photolysis and by xenon photosensitization. The quantum yield of the carbon dioxide decomposition was evaluated by Groth (5) as 0.98 for 1470 Å light. Carbon dioxide was photolysed at various pressures to determine the change of absorption of 1470 Å with pressure. After appropriate times, samples of the irradiated gas were removed from the reaction space by means of the Toepler pump, and analysed for carbon dioxide and carbon monoxide on a Blacet–Leighton micro gas analysis apparatus (1). From these data, the amount of light absorbed at each pressure was calculated. Some typical data are given in Table I, obtained with the lamp containing about 1 mm. of xenon, and operated at 120 ma.

TABLE I

Run No.	Carbon dioxide pressure, mm. mercury	Light absorbed quanta per sec.
1	0.14	0.45×10^{14}
2	0.36	0.41 " " "
3	5.4	8.5 " " "
4	14.1	10.8 " " "

From these data it is seen that pressures of several centimeters would have to be used to ensure complete absorption of all the 1470 Å light. However, use of pressures this high would necessitate very long runs in order that enough decomposition would occur to make the gas analyses significant.

Accordingly, the xenon photosensitized decomposition of carbon dioxide was investigated. With the lamp operating under the same conditions as

for the photolyses, runs were made at lower carbon dioxide pressures, but with small percentages of xenon added. The results of two such runs are given in Table II.

TABLE II

Run No.	Carbon dioxide pressure, mm. mercury	Xenon pressure, microns	Light absorbed, quanta per sec.
5	0.84	40	12.5×10^{14}
6	0.90	1.1	10.3×10^{14}

Thus it is seen that a very low pressure of xenon will absorb all of the 1470 Å radiation and decompose the carbon dioxide.

The data of Runs 1 to 4 fit the equation:

$$I_0 - I_{abs} = I_0\, e^{-0.270\, P},$$

where I_0 = incident intensity, 11.1×10^{14} quanta per sec.
I_{abs} = absorbed radiation in quanta per sec.
and P = pressure in millimeters of mercury.

The average intensity for Runs 5 and 6 is 11.4×10^{14} quanta per second. Since this figure agrees so well with the value of I_0 calculated for Runs 1 to 4, it is concluded that the quantum yield for the photosensitized decomposition is the same as for the photolysis.

One reason for assuming that the radiation from this source was predominantly 1470 Å was the fact that no solid deposit was observed in any of these experiments. If some of the 1295 Å line had been getting through, some carbon would have been formed (3).

Although the operating characteristics of this lamp have not been completely studied, it was found that with a filling of 1 mm. xenon and 4 mm. neon, the output at 120 ma. was 9.8×10^{14} quanta per second, while at 240 ma. it was 8.5×10^{14} quanta per second.

Acknowledgment

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LIGHT-SCATTERING STUDIES OF SODIUM THYMONUCLEATE[1]

By D. B. Smith[2] and H. Sheffer[3]

Abstract

The scattering of light by aqueous solutions of three different samples of sodium thymonucleate has been investigated. It was found that one sample in water consisted of randomly coiled molecules of molecular weight greater than 24×10^6. A second sample gave a less viscous solution in water and was only partially coiled, the coiling of the molecules being increased by the addition of a small amount of sodium chloride. In dilute salt solution the molecular weights of all three samples were greater than 3.7×10^6. Two samples of nucleate were degraded in solution by irradiation with ultraviolet light in the presence of hydrogen peroxide. In both cases the nucleate was converted into material consisting of rodlike molecules, 2400Å in length and having a molecular weight of 750,000.

Nucleic acids have received considerable attention because of their importance as constituents of cell nuclei. Measurements of the molecular dimensions of sodium thymonucleate have been made by several investigators; the methods used have been measurements of sedimentation and diffusion and of intrinsic viscosity and streaming birefringence. Molecular weights varying from 200,000 to 1,500,000 and axial ratios ranging from 120 to 400 have been reported (see discussion below). Recently a molecular weight of 35,000 has been estimated from dielectric dispersion measurements (15). It is well known that the physical state of sodium thymonucleate depends upon the method used for its isolation, and even the same procedure in the hands of a single investigator may yield products of varying properties (2). It is also possible that an estimate of molecular size depends upon the method used for obtaining it. For this reason it was considered that light-scattering measurements would be of interest.

Two samples of sodium thymonucleate which were previously described (2) and another prepared in the Department of Biochemistry, University of Toronto, were submitted to light-scattering studies in the Defence Research Chemical Laboratories, Ottawa.

Experimental

Preparation of Sodium Thymonucleate

The sodium thymonucleate batches were prepared from calf thymus nucleoprotein isolated by the method of Mirsky and Pollister (20). The protein was removed by repeated emulsification with chloroform – octyl alcohol (23) and the thymonucleate was precipitated with alcohol. The salt solutions used contained sodium citrate (0.01 *M*) to inhibit the action of desoxyribonuclease (17). All work was done in a cold room.

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It has been found by experience with a large number of batches that this procedure consistently yields products of higher intrinsic viscosity than does the method of Hammarsten (13).

Preparation of Solutions

Solutions of sodium thymonucleate of concentration 0.02 to 0.05% were made up by allowing the material to disperse in volumetric flasks for several hours at 5°C. Moisture determinations were made by drying to constant weight in a vacuum desiccator over phosphorus pentoxide at room temperature. Values varying from 13 to 18% were obtained.

For the light-scattering studies, the solutions were clarified by high-speed centrifugation at a low temperature and then treated in the manner described by Hadow, Sheffer, and Hyde (12). Water, substantially free from dust particles, was obtained by repeated distillations *in vacuo* in an apparatus originated by Martin (19). Sodium chloride solutions were clarified by prolonged high-speed centrifugation and were pipetted into the water in the light-scattering cell when required. Some samples of nucleate were degraded in solution by adding hydrogen peroxide (Merck's "Superoxol") to a concentration of 0.15% and irradiating in a Pyrex vessel for 40 to 55 min. with a General Electric 250 watt UVIARC lamp. This treatment markedly reduces the viscosity of thymonucleate solutions (3), and there is no recovery of the viscosity over 24 hr.

Viscosity Measurements

Even very dilute solutions (0.01%) of sodium thymonucleate exhibit marked anomalous viscosity, so that extrapolations to infinite dilution to obtain the intrinsic viscosity are difficult to make and give different values for different viscometers. An attempt was made to use a modified Goodeve (9) concentric cone viscometer so that extrapolations to zero rate of shear could also be made, but this too gave unsatisfactory results. For this reason, as a basis for comparison only, the batches were characterized by obtaining the apparent relative viscosities of 0.05% solutions, using a standard Ostwald viscometer (maximum rate of shear with water about 2300 sec.$^{-1}$). These values are given in Table I.

TABLE I

RELATIVE VISCOSITIES OF 0.05% SOLUTIONS

Batch No.	η_{rel} (in H_2O)	η_{rel} (in 0.02 *M* NaCl)	η_{rel} (in 0.02 *M* NaCl and 0.15% H_2O_2) after u.-v. irradiation
XIII	6.53	2.65	1.19
XI	4.45	2.38	1.19
XIX	3.08	1.84	

TABLE II

LIGHT-SCATTERING DATA

Run No.	Batch No.	Solvent	5461Å, $c \rightarrow 0$			4358Å, $c \rightarrow 0$			M_w	L, Å
			c/τ	$I_{60°}/I_{120°}$	$I_{33°}/I_{147°}$	c/τ	$I_{60°}/I_{120°}$	$I_{33°}/I_{147°}$		
1	XI	Water	0.51	2.0	3.6	0.26	2.0	3.8	$>5 \times 10^6$	>6000
2		"	0.45	2.0	3.7	0.22	2.0	3.9		
3		0.02M NaCl	0.83	2.1	5.0	0.47	2.1	5.2	$>4.4 \times 10^6$	>6000
11		"	0.75	2.1		0.40	2.1	5.3		
4		0.02M NaCl + 0.15% H_2O_2 Irradiated	0.445*	1.55	2.3	0.205*	1.6	2.4	760,000	2400
8	XIII	Water	0.51	3.2	11.4	0.26	3.1	11.6	$> 24 \times 10^6$	>9000
12		"	0.36	3.5	11.1	0.19	3.7	11.5		
5		0.02M NaCl	0.81	2.0	5.4	0.45	2.0	5.5	$> 4.3 \times 10^6$	>6000
6		"	0.75	2.1	5.2	0.41	2.0	5.2		
10		"	0.76	2.0	4.7	0.42	1.95	4.6		
7		0.02M NaCl + 0.15% H_2O_2 Irradiated	0.46*	1.5	2.3	0.205*	1.55	2.4	740,000	2400
9	XIX	0.02M NaCl	0.84	2.0	4.5	0.44	2.0	4.5	$> 3.7 \times 10^6$	>6000

* *Corrected for dissymmetry.*

Light-scattering Measurements

Light-scattering apparatus B described by Hadow, Sheffer, and Hyde (12) was used in these experiments. Benzene was used as the turbidity standard. The 5461Å and 4358Å lines were used. Dissymmetries (ratios of forward to backward intensities at equal angles about 90°) were obtained for the pairs of angles 60° and 120°, and 33° and 147°. The use of the equipment, the handling of solutions, and the difficulties involved in the use of aqueous solutions have been described in detail (12).

Measurements were made on three different batches of sodium thymonucleate in water, in 0.02 *M* sodium chloride solution and in the latter with 0.15% hydrogen peroxide added and then irradiated as described previously. Representative experimental results are shown in Figs. 1 and 2. The numbers on the plots refer to the run numbers in Table II, where the conditions employed and extrapolated values of c/τ (c = concentration corrected for moisture content, τ = turbidity) and the dissymmetry ratios are given. In Fig. 1 the concentrations have not been corrected for the moisture content of the nucleates, and dissymmetry corrections have not been applied to the turbidities.

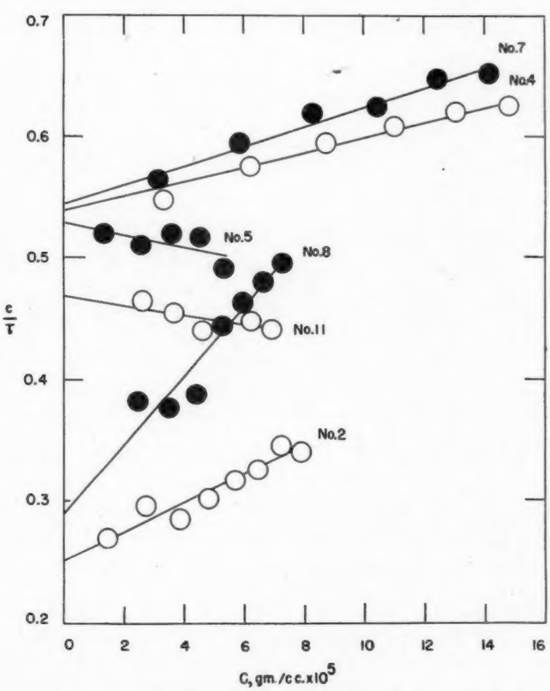

FIG. 1. *c/τ plotted against concentration*, λ = 4358Å.

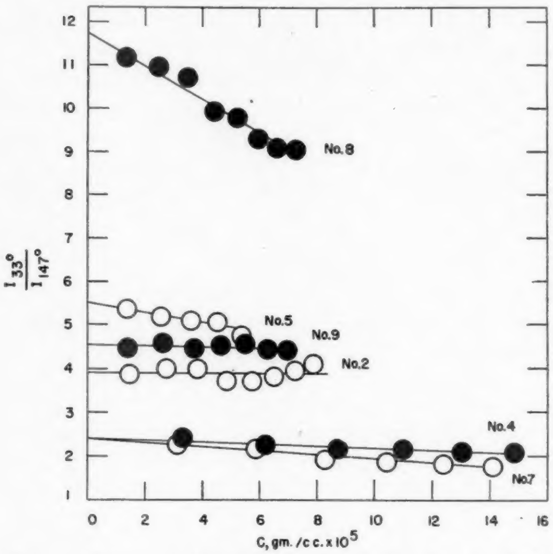

FIG. 2. *Dissymmetries plotted against concentration,* $\lambda = 4358$ Å.

The fluctuations encountered in individual runs can be assessed from the graphs, while the order of reproducibility between different runs using the same conditions can be seen in Table II. Most of the discrepancies can be traced to the difficulties involved in getting optically clean water and aqueous solutions (12).

Depolarization measurements were also made, but were rather inaccurate because of the very dilute solutions and the large particles involved and because of the effect of foreign particles which are usually present in the aqueous solutions. Some of the values are given below in the discussion.

Discussion

Oster (21) has shown that for large values of $\frac{L}{\lambda_1}$ (L = length of molecule, λ_1 = wave length of light in the medium), the dissymmetry ratio approaches limiting values equal to $\cot\frac{\theta}{2}$ for rods and $\cot^2\frac{\theta}{2}$ for random coils. For the angles used in this work the limiting values are for rods, $\frac{I_{60°}}{I_{120°}} = 1.73$ and $\frac{I_{33°}}{I_{147°}} = 3.38$ and for coils $\frac{I_{60°}}{I_{120°}} = 3.0$ and $\frac{I_{33°}}{I_{147°}} = 11.4$. Reference to Table II shows that the only solutions exhibiting dissymmetries below the limiting values for rods are the irradiated ones in Runs 4 and 7. Within experimental

error, the dissymmetries for Batch XIII in water (Runs 8 and 12) lie in the limiting region for random coils. The dissymmetries for all the other runs lie *between* the limiting values for rods and coils but are closer to the former, indicating partial coiling of the molecules. The fact that the dissymmetries for the green and the blue lines are the same, within experimental error, indicates that the molecules are longer than the wave length of the light used, since the curves of dissymmetry against $\frac{L}{\lambda_1}$ flatten out in this region (21).

The dissymmetries for Runs 4 and 7 are nearly the same and fit the theoretical curves for rods (18, 21) quite well, giving a value for the length of the rod $L = 2400$Å. The correction factors for the 90° turbidities can also be obtained from theoretical curves of correction factor plotted against dissymmetry (18). Using these values, together with $\left(\frac{c}{\tau}\right)_{c\to 0}$ and $\frac{\partial n}{\partial c} = 0.160$ (26) (n = refractive index), the weight-average molecular weights M_w(given in the second last column of Table II) can be calculated using the Debye equation (18). The correction factors increase very sharply as the limiting value of dissymmetry is approached, so that only an estimate of the minimum value can be obtained for large molecules. Similarly only an estimate of the minimum length can be obtained from the present data for all the runs other than 4 and 7.

The limiting value of the dissymmetry, when plotted against correction factor, is approached asymptotically. However, a value of 40 for the minimum correction factor for coils was estimated where $\frac{I_{33^\circ}}{I_{147^\circ}} \to 11.4$, and a similar value of 7 for rods was estimated where $\frac{I_{33^\circ}}{I_{147^\circ}} \to 3.38$. The minimum correction factors for the partially coiled molecules were obtained by dividing the difference between these minimum values (7 and 40) in the same ratio as the measured dissymmetry divides the difference between the theoretical limiting dissymmetries for rods and coils (3.38 and 11.4), and adding this value to 7. The minimum values of M_w given in Table II could then be calculated.

Batch XI in water is partially coiled, but when sodium chloride is added the coiling increases, as evidenced by the higher dissymmetry. The addition of sodium chloride obviously makes the solvent a "poorer" one since it has been shown many times that the more favorable the solvent the more extended the polymer molecule. Thus the addition of sodium chloride would be expected to lower the relative viscosity, which is the case as shown in Table I. This explanation is, however, complicated by the simultaneous suppression of the electroviscous effect, since this also contributes to the reduction in the relative viscosity (1; 5, p. 523).

The Huggins constant μ_1, which is related to the extent of solvent–polymer attraction, can be calculated from the slopes of the lines in Fig. 1 (18). Precipitation takes place in systems where μ_1 approaches a value of 0.55, while lower

values of μ_1 indicate a more favorable solvent. μ_1 has a value of 0.50 when the slope is zero, higher values for negative slopes, and lower values for positive slopes. It is thus evident that the solvent is more unfavorable in Runs 5 and 11 (salt solutions) than in 2 and 8 (aqueous solutions). The positive slope for Run 4 indicates that the molecules of lower molecular weight are more soluble. Similar effects for other polymer systems have been discussed in detail by Sheffer (24).

In the case of Batch XIII, the molecule is so large that it is randomly coiled owing to folding of the chains. Gulland, Jordan, and Taylor (11) have postulated the formation of hydrogen bonds between purine–pyrimidine hydroxyl groups and some of the amino groups. Such bonds are relatively weak, so that addition of sodium chloride in this case, tending to cause further coiling, actually brings about the rupture of some of these bonds, resulting in molecules which are very much like those of Batch XI in sodium chloride solution. Creeth, Gulland, and Jordan (6) conceived the possibility of the rolling up of a single polynucleotide chain following the fission of hydrogen bonds between nucleotides in that chain and also considered the possibility of rupturing of such bonds between adjacent chains.

Irradiation of both these batches in the presence of hydrogen peroxide provides hydroxyl radicals which degrade the molecule considerably (3). The resulting smaller molecule does not exhibit a tendency to coil up even in sodium chloride solution, but is rodlike in shape and has a molecular weight and length of the order of magnitude found with the use of other techniques for sodium thymonucleate prepared by this and other methods.

The depolarization measurements were not accurate enough to warrant a complete tabulation. Representative results are : for Run 8—$\rho_u = 0.04$, $\rho_v = 0.007$, $\rho_h = 0.2$; for Run 2—$\rho_u = 0.03$, $\rho_v = 0.013$, $\rho_h = 0.5\text{-}0.7$; for Run 4—$\rho_u = 0.035$, $\rho_v = 0.010$, $\rho_h = 0.35\text{-}0.45$. For the runs on unirradiated material in salt solution ρ_u was in general lower, ρ_v was very low, and ρ_h could not be accurately evaluated. Based on the discussions of Doty and Kaufman (7) and Doty and Stein (8), these results are consistent with the facts that the nucleate in water consists of very large anisotropic molecules and that in salt solution the tendency is for the molecules to become more isotropic.

The general trend of the results from the light-scattering work is in agreement with the viscosity values given in Table I. Higher molecular weights and greater extension of the molecule result in higher relative viscosities. The good agreement obtained for the molecular weights and lengths of the molecules of the irradiated samples is further supported by the identical values for the relative viscosities of their solutions.

Greenstein and Jenrette (10) postulated, on the evidence of viscosity measurements, that reversible depolymerization takes place on addition of neutral salts. It would appear, however, that a large part of this effect is more likely to be due to coiling and uncoiling of the molecules rather than to reversible depolymerization. Jordan and Oster (14) have shown on the basis of

light-scattering experiments that the rodlike protein actomyosin is partially coiled in potassium chloride solutions and that the addition of adenosine triphosphate increases the coiling of the molecules.

For purposes of comparison, Table III lists values of molecular weights and axial ratios obtained by other investigators and in the present study. The low value of 35,000 obtained from dielectric dispersion measurements (15) is probably due to the fact that this method indicates only the size of the rotating unit which may be smaller than the molecule. The coiling of the molecules in salt solution could be used to explain some of the other dielectric phenomena observed (15).

TABLE III

MOLECULAR WEIGHT AND SIZE OF SODIUM THYMONUCLEATE MOLECULES

Ref. to method of nucleate preparation	Method	Reference	Mol. wt.	Axial ratio $\frac{a}{b}$	b, Å	a, Å
(13)	Visc. and str. birefringence	(25)	500,000–1,000,000	300		6000–4500
(13)	Sedimentation and diffusion	(22, p. 443)	200,000			
(13)	"	(26)	430,000–580,000	170–400	13–16	2720–5200
(13)	"	(16)	1,500,000	284	18.4	5226
(20 & 23)	"	(4)	820,000	120		
(13)	Dielectric measurements	(15)	35,000			
(20 & 23)	Light scattering	This investigation—degraded material	750,000	(ca. 140)		2400
(20 & 23)	"	This investigation — unirradiated material	>3,700,000–>24,000,000			

It can be seen from Table III that the estimates of molecular size obtained in the present work for unirradiated material are considerably greater than any values recorded previously. It might be thought that this is due to the presence of a gel component similar to that found by Cecil and Ogston (4) from sedimentation experiments. However, no such component was found when dilute solutions (0.015 to 0.06%) of Batch XI in 0.02 M sodium chloride were centrifuged at 59,780 r.p.m. in a "Spinco" ultracentrifuge. The boundary was a single, very sharp spike similar to that reported by Kahler (16). Sedimentation and diffusion methods are very good for globular materials but their application to the determination of molecular weights of large, very asymmetric molecules is not on a firm basis. Other methods that have been applied to nucleates are also open to criticism. The light-scattering data for the degraded material should be accurate to within ±10%, as no limiting values are involved. Since the viscosity values for the unirradiated nucleates are much higher than for the degraded material it would appear that the high molecular weights obtained for the former are quite reasonable.

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OSMOTIC PRESSURE, MOLECULAR WEIGHT, AND VISCOSITY OF SODIUM ALGINATE[1]

BY F. G. DONNAN[2] AND R. C. ROSE[3]

Abstract

This work shows how the molecular weight of sodium alginate can be determined by measuring the values of the osmotic pressure, P, in the presence of low concentrations of sodium chloride, and extrapolation of P/C to $C = 0$, where C is the concentration of sodium alginate. For different samples of sodium alginate the slope of the curve P/C against C was independent of the molecular weight. Molecular weight values from 48,000 to 186,000 were obtained. The intrinsic viscosity of the different samples were linearly related to the degree of polymerization.

Introduction

Sodium alginate is extracted from brown seaweeds; one method is to heat ground seaweed in sodium carbonate solution, filter the extract, and precipitate sodium alginate by adding alcohol. A commercial practice is to precipitate calcium alginate by adding calcium chloride solution to the extract; the calcium alginate is then bleached with hydrochlorite, converted to alginic acid by extracting with hydrochloric acid, and then to sodium alginate by neutralizing the alginic acid with sodium hydroxide.

Alginic acid is a polymer of β-d-mannuronic acid (9). Its structure is comparable with that of cellulose although alginic acid is more contracted; its period along the fiber axis is 8.7Å whereas that of cellulose is 10.3Å (2). Alginic acid has carboxyl groups in place of the alcohol end-groups of cellulose; it resembles oxycellulose in chemical and physical properties. The salts of alginic acid, such as sodium alginate, apparently exist in a hydrated form containing a molecule of water per mannuronate unit (4). Sodium alginate is water soluble and the viscosity of solutions of equal concentration varies widely with weed origin and severity of the extraction process. The viscosity of cellulose is closely related to its molecular weight (11); one object of this investigation was to determine if viscosity and molecular weight of sodium alginate were similarly related. Of the various viscosity terminologies intrinsic viscosity, $[\eta]$, appears to be the most useful. It is defined by the equation

$$[\eta] \equiv \lim_{C \to 0} \left(\frac{\eta_r - 1}{C} \right) \equiv \lim_{C \to 0} \left(\frac{\ln \eta_r}{C} \right),$$

where η_r is the viscosity of the solution relative to that of the solvent and C, the concentration in grams per 100 ml. of solution (10, 11). Intrinsic viscosities were determined in this study.

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Contribution from the Sir William Ramsay and Ralph Forster Laboratories of Chemistry, University College, London, England. This paper constitutes part of a thesis submitted to the University of London, in 1937, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Another object was to use osmometry as a means of estimating molecular weights of colloidal electrolytes—or polyelectrolytes as they have recently been called. For nonelectrolytes such as cellulose nitrate (6), cellulose acetate (5) and polyvinyl acetate (14), osmometers have proved satisfactory and for a given sample the osmotic pressure per unit of concentration is independent of the solvent, provided this quantity is extrapolated to zero concentration; at finite concentrations the osmotic pressure may be much higher in one solvent than in another. Polyelectrolytes may ionize extensively and it is obvious that the osmotic pressure of a completely ionized substance depends not on the number of macromolecules present but on $(n + 1)$ times this number, where n is the number of ions of opposite charge (sodium ions in the system under study) per macromolecule. For high polymers, $(n + 1)$ would approximate n, and the limiting value of the osmotic pressure per unit of concentration would be an approximate measure of the equivalent weight; in the system under study, the weight of a single sodium mannuronate unit. Under these conditions the limiting value of the osmotic pressure per unit of concentration is almost independent of the degree of polymerization.

Application of the Donnan (or Gibbs–Donnan) theory of membrane equilibrium (7, 8) shows that it is possible to counterbalance partially the osmotic pressure due to the oppositely charged ions by adding a freely diffusible electrolyte, such as sodium chloride. The diffusible electrolyte sets up a counter-osmotic pressure because of the unequal ionic distribution across the membrane when the system is at equilibrium. Counterbalancing is complete only at or near the limit $C \rightarrow 0$, and to obtain a reliable value for the molecular weight, the osmotic pressure per unit of concentration must be extrapolated to zero concentration. Provided aggregation is avoided, the limiting value of the osmotic pressure per unit of concentration should be independent of the sodium chloride concentration. The mean molecular weight M is then calculated from the equation

$$M = \frac{RT}{\lim\limits_{C \to 0} P/C}$$

$$= \frac{253{,}000}{\lim\limits_{C \to 0} P/C} \text{ at } 25^\circ\text{C.},$$

where P is the pressure expressed in centimeters of water at 4°C. and C is the concentration of sodium alginate in grams per 100 ml. solvent.

Materials and Methods

The sodium alginate was supplied by Messrs. Alginate Industries Limited, London, who extracted it from *Laminaria Cloustoni* stems. The samples were washed with alcohol to remove traces of salts, and air dried. Dry matters were determined by drying portions to constant weight over phosphorus pentoxide in vacuum. Solutions were prepared on a dry weight–volume of solution basis.

It would have been better for osmotic pressure studies to have expressed the concentration in grams per 100 ml. of solvent (7, 8) but the error introduced by using grams per 100 ml. of solution is not significant. The water used in preparing alginate solutions and salt solutions was saturated with thymol to inhibit biological decomposition of the alginate.

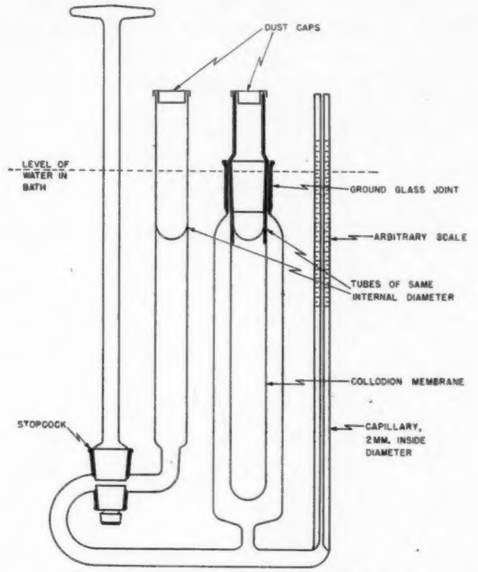

FIG. 1. *Osmometer (total height 12 in.).*

The osmometer employed is shown in Fig. 1 and was a modification of one developed earlier in these laboratories by Oakley (13) for low osmotic pressures. It had the advantages of a capillary osmometer without the disadvantage of having to apply a capillary correction. Collodion membranes were used; they were made by pouring two coats of 4% pyroxylin in 60–40 anhydrous ether–absolute alcohol (by volume) on the outside of a slowly revolving horizontal test tube, which had a pinhole in the end (1). When dry, the membrane was removed by forcing water through the pinhole, trimmed to length, slipped on the osmometer tube, and tied with linen thread. Osmotic pressures were determined at 25°C. When the osmometer was to be used, it was filled with salt solution and the membrane, containing alginate and salt solution, was fitted in place. To extend the membrane, the stopcock was opened and the level of the liquid in the open tube was reduced well below that of the solution in the membrane. The stopcock was then closed. The capillary was wiped internally with a small piece of soap on the end of a fine wire to avoid any sticking of the liquid in the capillary. The instrument was read as follows: the level of liquid in the capillary was read on an arbitrary scale; the stopcock was opened and the level of the liquid in the open tube raised or lowered by means

of a pipette until the liquid in the capillary was at its original level; the difference between the level of the liquid in the membrane and in the open tube was determined with a precision cathetometer and recorded as the osmotic pressure. (Density corrections for salt concentration and temperature were applied.) Experience showed that with the type of membrane used, a three day period was ample for the system to reach equilibrium; readings were taken at intervals of not less than 24 hr. after this time and again 24 hr. later if the first two readings failed to agree within 0.03 cm. This degree of reproducibility was also obtained with the same solutions in different osmometers.

Viscosities were determined at 25°C. in B.S.I. Ostwald viscometers.

Experimental

Effect of Sodium Chloride on the Limiting Osmotic Pressure per Unit of Concentration

As stated previously, the limiting value of the osmotic pressure per unit of concentration should be independent of the salt concentration. To test this the osmotic pressures of two to five concentrations of one sample of sodium alginate in each of five concentrations of salt and of another sample in each of two concentrations of salt were measured. The results are shown in Fig. 2

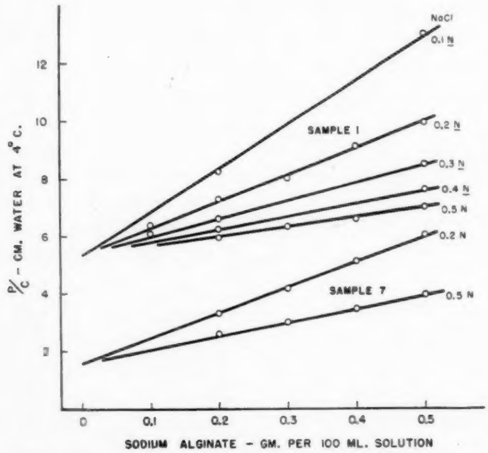

FIG. 2. *Effect of sodium chloride concentration on the osmotic pressure of sodium alginate.*

where the osmotic pressure per unit of concentration is plotted against the concentration. They show that there was a linear relation between osmotic pressure per unit of concentration and concentration, and that for each sample of sodium alginate the limiting osmotic pressure value was independent of the concentration of sodium chloride.

Osmotic Pressure and Mean Molecular Weight

In this study the osmotic pressures of four or five concentrations of each of seven samples of sodium alginate, all in $0.2N$ sodium chloride, were measured. The results are shown in Fig. 3 and data from these results are given in Table I.

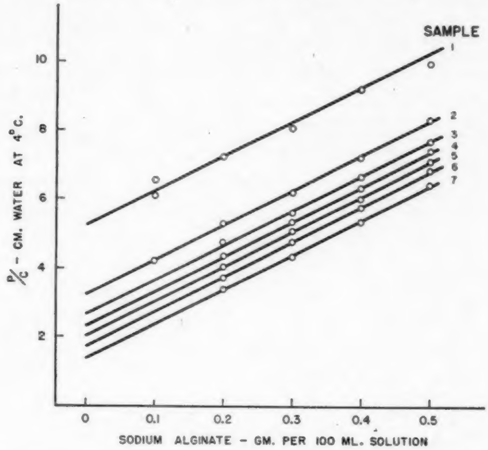

FIG. 3. *Osmotic pressure of different samples of sodium alginate in 0.2N sodium chloride.*

TABLE I

THE MEAN MOLECULAR WEIGHT OF SODIUM ALGINATE

(Common $\frac{dP/C}{dC}$, 9.57)

Sample	Slope $\frac{dP/C}{dC}$	$\lim_{C \to 0} P/C$	Molecular weight	Degree of polymerization	Anhydrous molecular weight
1	9.19	5.250	48,000	220	44,000
2	9.15	3.018	84,000	390	77,000
3	9.41	2.678	95,000	440	87,000
4	9.99	2.292	110,000	510	101,000
5	9.77	2.048	123,000	570	113,000
6	10.52	1.755	144,000	665	132,000
7	9.51	1.362	186,000	860	170,000

Among the seven samples the slope of the curves varied from 9.15 to 10.52, but statistically, as each slope was based on only four or five points, this variation is not significant. Consequently, the common slope for all the values was calculated and used to determine the limiting value of P/C as $C \to 0$. The mean molecular weights were calculated by means of the equation given previously and they varied from 48,000 to 186,000. The equivalent weight of sodium alginate, based on its structure (9) is 198 but, as stated previously, sodium alginate may be hydrated. Analyses of samples which had been dried as previously described showed them to have an equivalent weight of 216.

This corresponds to the hydrated form and this value of 216 was used in calculating the degree of polymerization (D.P.), ie., the number of sodium mannuronate units per macromolecule. The D.P. values, shown in Table I, ranged from 220 to 860. The molecular weight values, corrected for the molecule of water, are also shown in Table I.

Viscosities of Sodium Alginate Solutions

As stated previously the viscosities of sodium alginate solutions of equal concentrations vary with weed origin and severity of the extraction process. To obtain reliable viscosity values for sodium alginate the effects of salt, hydrogen ion concentration, and rate of shear had to be determined. The results showed that sodium alginate exhibited the well known electroviscous effect (12, p. 171); $0.1N$ sodium chloride swamped this effect, and higher concentrations, up to $0.5N$ sodium chloride, either had no additional effect or slightly increased the viscosity of 0.1 to 0.5% solutions of sodium alginate. Between pH 6 and 8 the viscosities of 0.5% solutions of Sample 4 were independent of pH and decreased during storage at 25°C. less than 1% per week—suggesting slight depolymerization. Outside this pH range the viscosity decreased more rapidly. Increasing the rate of shear had little effect on the viscosities of solutions of the lower polymers but it decreased the viscosities of the higher polymers. The effect was more pronounced at higher concentrations of sodium alginate but changing from one viscometer to the next in the B.S.I. series of viscometers had little effect below viscosities of 40 centipoises.

Viscosity–concentration curves for each of the seven samples used previously were determined and as $0.1N$ sodium chloride had proved sufficient to suppress the electroviscous effect it was used throughout. The results for five samples are shown in Fig. 4. These curves are all similar and can be made to

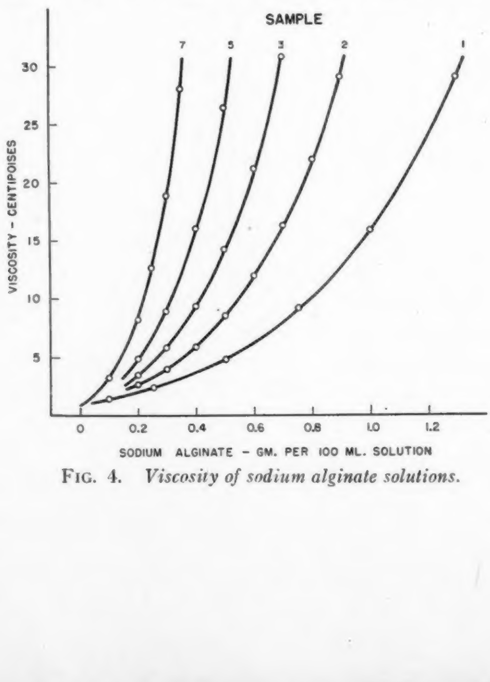

FIG. 4. *Viscosity of sodium alginate solutions.*

coincide throughout their length by selecting a suitable horizontal scale for each one. Thus if the scale for Sample 3 is multiplied by 1.9 the curve coincides with that for Sample 1. The factors by which the horizontal scales have to be multiplied to make curves coincide are referred to as "relative concentration factors". The latter for all the samples were calculated relative to 4.1 for Sample 1, and are recorded in Table II.

TABLE II

MOLECULAR WEIGHT AND VISCOSITY OF SODIUM ALGINATE

Sample	Degree of polymerization	Intrinsic viscosity	Relative concentration factor	$\frac{D.P.}{[\eta]}$
1	220	4.2	4.1	52
2	390	5.7	5.9	68
3	440	7.8	7.8	56
4	510	9.1	8.9	56
5	570	10.0	10.1	57
6	665	12.0	12.0	55
7	860	14.4	14.3	60
			Mean	58

Intrinsic viscosities, as defined previously, were calculated and are also recorded in Table II. Neither $\frac{\eta_r - 1}{C}$ nor $\frac{\ln \eta_r}{C}$ was linearly related to C; both curves for each sample were therefore plotted on the same graph and extrapolated to a common point at zero concentration to give the best limiting value.

Relation Between Molecular Weight and Viscosity

The molecular weight data and the viscosity data are recorded in Table II and the intrinsic viscosities are plotted against molecular weights in Fig. 5. The correlation coefficient between these two properties was .98.

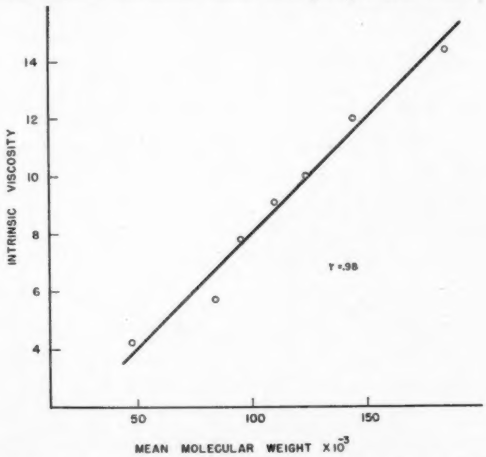

FIG. 5. *Relation between molecular weight and intrinsic viscosity of sodium alginate.*

Summary and Discussion

This work shows that, as postulated by the Membrane Equilibrium theory, the limiting osmotic pressure per unit of concentration as $C \rightarrow 0$ is independent of the concentration of diffusible electrolyte. If each concentration of sodium chloride is regarded as a different solvent this is comparable with work on cellulose nitrate (6), cellulose acetate (5) and polyvinyl acetate (14), which showed that the limiting values were independent of the solvent used. For different samples of sodium alginate the slope of P/C against C was independent of the molecular weight, which agrees with theoretical calculations and actual measurements on solutions of nonelectrolyte polymers in organic solvents (10, 14). The negative correction to P/C introduced by Huggins (10) was not applied in this work and its effect would be to decrease the slope of the curves slightly but it would have no measurable effect on the limiting value.

Mean molecular weights between 48 000 and 186,000 were obtained representing degrees of polymerization of 220 to 860 sodium mannuronate units. These are not the extremes: on the low side, gentle acid treatment will depolymerize alginic acid so far that it will diffuse through a membrane when redissolved as sodium alginate; on the high side, preparations more viscous than any studied in this investigation have been extracted, but the most viscous sample depolymerized slowly (as shown by viscosity measurements) during storage and was not ideal for osmotic pressure studies.

The relation between intrinsic viscosity and molecular weight is approximately linear and therefore fits Staudinger's equation as well as the more general one for homogeneous samples attributed to Mark (10), $[\eta] = kM^v$, where k and v are empirical constants. Agreement with these equations does not imply that the samples of sodium alginate were homogeneous. Debye and Bueche (3) stress that the relation between intrinsic viscosity and molecular weight is rather indirect and depends essentially on the type of polymer molecule under consideration.

The mean value of $\frac{D.P.}{[\eta]}$ was 58. The average value for various derivatives of cellulose in organic solvents and cellulose in cuprammonium solution was 260 (11). A comparison suggests that the product of the spherical volume and the shielding ratio (3) of molecules of the same degree of polymerization is considerably higher for sodium alginate than for cellulose, but this may be due to the ionization of sodium alginate and not to any differences in chain structure.

The relative concentration factors are obviously closely related to the intrinsic viscosities; differences may be due to errors in estimating the two quantities. The concentration factors represent the relative volumes of solutions (in $0.1N$ sodium chloride) of the same viscosity that can be prepared with the same amount of each sample. The reciprocals of these factors represent the relative concentrations of each sample required to give solutions of the same

viscosity. This method of comparing concentrations at the same viscosity is similar to that used in reporting gel strengths of agar in which the concentration of agar necessary to give a specified gel strength is recorded.

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Some Observations on the Nitration of Vanillin

The preparation of 5-nitrovanillin, by the direct nitration of vanillin, has been accomplished by several workers. Bentley (1) reported a 50% yield using concentrated or fuming nitric acid; Simonsen and Rau (6) a 62% yield by the use of mixed oxides of nitrogen, and Francis (3) quantitative yields by the nitrating action of benzoyl nitrate. Other preparations have been described (2, 4, 5, 7) but in these cases no yields were reported. It has now been found possible to prepare this compound in high yields by a procedure similar to that of Simonsen and Rau. Nitrating oxides, generated by the action of sulphuric acid on sodium nitrite were passed into an ice-cooled, ethereal solution of vanillin. The required product separated on standing. Modifications introduced included the passage of air into the reaction mixture along with the nitrating gases, and the use of concentrated, instead of dilute, sulphuric acid for the generation of the nitrogen oxides from sodium nitrite. It would appear that the main effect was the production of a larger percentage of nitrogen dioxide as evidenced by the permanent reddish-brown gases produced in these cases.

TABLE I

PREPARATION OF 5-NITROVANILLIN

Vanillin, gm.	Ether, ml.	Vol. of flask, ml.	Reaction time, hr.	Yield, %
10	300	500	3	24*
10	300	500	3	100†
100	2000	3000	2	60†
60	1200	2000	3	39†
10	200	500	3	63†
10	300	500	3	90‡
10	300	500	3	90‡
35	700	1000	1	84‡
10	300	500	3	89‡§

**Dilute sulphuric acid used to generate the nitrating gases.*
†*Air admitted along the with the nitrating gases prepared using dilute sulphuric acid.*
‡*Concentrated sulphuric acid used to generate the nitrating gases; reaction mixture well agitated.*
§*Small glass beads (135 gm.) of average diameter 4 mm. added to the mechanically stirred reaction mixture.*

However, as may be seen from Table I, the wide variation in the yield of the 5-nitrovanillin cannot very well be explained on these bases only. It is suggested that a further explanation is that the reaction is catalyzed by glass surfaces, in support of which it was noted that:

1. There was generally an induction period before the commencement of precipitation from the main reaction mixture. This varied very markedly and

could not be explained by autocatalysis (seeding with the prepared crystals had no effect) nor by the time required to saturate the ether (the induction time was independent of the volume of ether).

2. The first formation of precipitate was almost instantaneous at the walls of the inlet tube, which rapidly became blocked even when tubes of diameter up to 15 mm. were used.

3. In one run, in which the inlet tube almost touched the bottom of the flask, the precipitate formed on the wall of the flask directly opposite the gas inlet, almost as rapidly as at the walls of the inlet tube.

4. Precipitation occurred also near the neck of the flask, even above the ether level, where the gases had to pass through a constriction before escaping.

5. When the reaction material stood overnight, heavy coatings of the precipitate formed over the walls of the vessel and the outer walls of the inlet tube.

6. Carrying out the reaction in the presence of glass beads resulted in the rapid production of a good yield of finely divided product, which possibly was first formed on the glass surfaces but then removed by the constant agitation.

In attempting to prepare the monoacetate of 5-nitrovanillin, the previously unreported* triacetate derivative was obtained and characterized as

$$3\text{-}OCH_3\text{-}4\text{-}OAc\text{-}5\text{-}NO_2\text{-}C_6H_2\text{-}CH(OAc)_2.$$

Experimental

5-Nitrovanillin.—The general procedure outlined by Simonsen and Rau (6) was followed without however the addition of water to the ethereal solution. The variations and results are given in Table I. Since no suitable solvent for recrystallization was found, purification was effected in the reported manner (6) by washing the precipitated material with excess ether, m.p., 176°C. (reported 175° to 176°C.).

Caution.—In recovering the ether from these runs, it was discovered that after most of the ether had been removed and the temperature of the residue began to rise an explosion took place, producing large quantities of yellow-orange smoke and filling the flask with carbon.

5-Nitrovanillin Triacetate.—5-Nitrovanillin (25 gm.) was suspended in acetic anhydride (50 ml.) and two drops of concentrated sulphuric acid added. From the resulting red-colored solution, a brownish precipitate was deposited. Hydrolysis of the residual solution with boiling water yielded further crude product. Combined yield, 42 gm. (97%), m.p. 104°C. Recrystallization from dilute acetic acid and from aqueous alcohol gave 5-nitrovanillin triacetate as very pale yellow needles, m.p. 121°C. Calc. for $C_{14}H_{15}O_9N$: OCH_3, 9.09; Ac, 37.9. Found: OCH_3, 8.7; Ac, 37.8.

* *Also independently prepared by C. D. Logan at McGill University, Montreal.*

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